

Retinoic Acid Can Directly Promote TGF- β -Mediated Foxp3⁺ Treg Cell Conversion of Naive T Cells

Daniel Mucida,¹ Karina Pino-Lagos,² Gisen Kim,¹ Elizabeth Nowak,² Micah J. Benson,² Mitchell Kronenberg,¹ Randolph J. Noelle,^{2,*} and Hilde Cheroutre^{1,*}

¹La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA

²Department of Microbiology and Immunology, Dartmouth Medical School and Norris Cotton Cancer Center, Lebanon, NH 03756, USA

*Correspondence: randolph.j.noelle@dartmouth.edu (R.J.N.), hilde@liai.org (H.C.)

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The article by Hill et al. (2008), published in the November 14, 2008 issue of *Immunity*, describes a mechanism by which retinoic acid (RA) enhances TGF- β -induced Foxp3 expression. The authors propose that RA does not act directly on naive T cells during activation in culture but rather indirectly via negative regulation of an accompanying population of effector or memory CD4⁺ CD44^{hi} cells. They reasoned that the increased generation of Foxp3⁺ cells in response to RA in culture, as described previously (Coombes et al., 2007; Elias et al., 2008; Mucida et al., 2007; Sun et al., 2007; Xiao et al., 2008), represented the lifting by RA of inhibition imparted by accompanying CD4⁺ CD44^{hi} T cells, rather than by direct or indirect effects of RA on the Foxp3 expression of the primed naive T cells themselves.

In order to assess the effects of RA on naive T cells in the absence of accompanying CD4⁺ CD44^{hi} T cells, we sorted (CD4⁺ CD25⁻ CD44^{lo} CD62L⁺) GFP⁻ T cells (more than 99.7% purity) from Foxp3-eGFP reporter mice (Figure S1A available online) by flow cytometry. After 4 days of stimulation with anti-CD3 and anti-CD28, we stained CD4 cells with 7AAD to exclude dead cells; additionally, forward and side scatter (area versus width) was used to exclude doublets, and we evaluated Foxp3 expression via GFP staining. Addition of RA enhanced Foxp3 induction more than 50% by use of 1 or 10 ng/ml doses of TGF- β (Figure S1B). Because the sorting efficiency is not 100%, it is possible that extremely low numbers of “accompanying” memory or effector cells could still influence these results. To exclude this possibility, we used FACS-sorted CD4⁺ CD25⁻ CD44^{lo} CD62L⁺ T cells, isolated from B7-1 and B7-2 double-deficient mice (*Cd80*^{-/-} *Cd86*^{-/-}), which even before sorting already contain less than 5% of memory or effector CD44^{hi} cells (data not shown).

RA also greatly enhanced Foxp3 induced by TGF- β in CD4⁺ CD25⁻ CD44^{lo} CD62L⁺ naive T cells isolated from *Cd80*^{-/-} *Cd86*^{-/-} mice (Figure S1C). Moreover, we showed previously that RA is able to counterbalance the inhibitory effects of costimulation on TGF- β -mediated Foxp3 induction, with either CD4⁺ CD25⁻ or CD4⁺ Foxp3⁻ T cells (Benson et al., 2007). To confirm these results, we used OTII TCR transgenic CD4⁺ CD25⁻ CD44^{lo} CD62L⁺ cells sorted by flow cytometry and tested the effects of RA by using increasing doses of anti-CD28 stimulation. We found that RA markedly enhanced TGF- β -mediated Foxp3 induction on pure naive CD4⁺ T cells that were stimulated with anti-CD3 and various doses of anti-CD28 (Figure S1D). The enhanced Foxp3 expression mediated by RA is more pronounced on naive monoclonal OTII TCR transgenic T cells as compared to polyclonal T cells, consistent with a lesser frequency of “contaminating” memory T cells.

Finally, because we showed previously that RA-mediated enhanced expression of Foxp3 is greatly reduced in the absence of IL-2 (Mucida et al., 2007), we investigated the effects of RA on naive T cells with various doses of exogenous IL-2. Although IL-2-deficient mice develop inflammatory disorders, *Il2*^{-/-} *Cd80*^{-/-} *Cd86*^{-/-} mice are healthy and, more importantly, they do not contain T regulatory cells. At steady state, ~99% of all CD4⁺ T cells isolated from *Il2*^{-/-} *Cd80*^{-/-} *Cd86*^{-/-} mice are naive (data not shown). The CD4⁺ T cells were further sorted by flow cytometry so that highly purified naive CD4⁺ CD25⁻ CD44^{lo} CD62L⁺ cells (more than 99.9% purity) were obtained. The sorted naive CD4⁺ *Il2*^{-/-} *Cd80*^{-/-} *Cd86*^{-/-} T cells were tested for TGF- β -induced Foxp3 expression in the presence of increasing doses of IL-2 and anti-CD3 and anti-CD28 coated beads, with or

without RA. The data showed that 1 nM RA distinctly enhanced TGF- β (1 ng/ml)-mediated Foxp3 induction in pure naive CD4⁺ T cells at all doses of IL-2 examined (Figures S1E and S1F). Strikingly, although the expression of Foxp3 was much reduced, RA enhanced TGF- β -mediated Foxp3 induction not only in the absence of memory or effector T cells but also in the absence of IL-2.

These data demonstrate that RA mediates enhanced TGF- β -induced Foxp3 expression upon activation of pure naive T cells in the absence of accompanying CD4⁺ CD44^{hi} T cells. In addition, we confirmed, as Hill et al. (2008) proposed, that RA also efficiently counteracts inhibitory effects of CD44^{hi} T cells on Foxp3 induction (data not shown), which indicates that RA is able to enhance Foxp3 expression both, via effects directly on the primed naive T cells as well as indirectly via inhibitory effects on accompanying CD4⁺ CD44^{hi} T cells.

There is no doubt that the new findings by Hill et al. (2008) add an important new pathway by which RA can enhance Foxp3 induction, which has been suggested previously (Elias et al., 2008; Mucida et al., 2007; Xiao et al., 2008). Nevertheless, published data, together with the data presented here, disagree with the central statement proposed by Hill et al. (2008) that the enhanced expression of TGF- β driven Foxp3 mediated by RA is an indirect effect that requires suppression of accompanying CD4⁺ CD44^{hi} T cells rather than via direct or indirect effects on the primed T cells themselves. Under physiological conditions, naive T cells may be exposed to cytokines and effector or memory cells, and hence it is likely that during priming of naive T cells, both mechanisms of RA-mediated enhanced TGF- β driven Foxp3 expression in the primed T cells will synergize in vivo. Therefore, elucidating and understanding both processes by which

RA affects naive and already differentiated T cells is important and may lead to the identification of possible targets for therapeutic interventions to treat various inflammatory and autoimmune diseases.

SUPPLEMENTAL DATA

Supplemental Data include one figure and can be found with this article online at [http://www.immunity.com/supplemental/S1074-7613\(09\)00147-2](http://www.immunity.com/supplemental/S1074-7613(09)00147-2).

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Response to Letter from Mucida et al.

Jonathan A. Hill,¹ Jason A. Hall,^{2,3} Cheng-Ming Sun,² Qi Cai,⁴ Norbert Ghyselinck,⁴ Pierre Chambon,⁴ Yasmine Belkaid,² Diane Mathis,¹ and Christophe Benoist^{1,*}

¹Section on Immunology and Immunogenetics, Joslin Diabetes Center; Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02215, USA

²Mucosal Immunology Unit, Laboratory of Parasitic Diseases, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

³Immunology Graduate Group, University of Pennsylvania, Philadelphia, PA 19104, USA

⁴Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université Louis Pasteur, 67404 Illkirch, France

*Correspondence: cbdm@joslin.harvard.edu

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We thank our colleagues for their interest in our recent paper, which showed that retinoic acid (RA), binding to the RAR α receptor, influences TGF β -induced Foxp3 expression through an indirect manner, either by repressing IL-6R α or by dampening the production of inhibitory cytokines (IFN- γ , IL-4, and IL-21). The source of these cytokines, in our culture conditions, was CD44^{hi} memory and/or effector phenotype CD4⁺ T cells. Several other papers have appeared on the topic, investigating the responsiveness of isolated naive cells to RA, a question of importance in understanding the molecular mechanisms that link RA to FoxP3 expression. This letter offers us the possibility to clarify the issue.

RA could influence FoxP3 induction through several, nonmutually exclusive, mechanisms. First, an immediate and “direct” effect leading to activation of the FoxP3 locus, acting in naive T cells independently of any other influence (e.g., by direct transactivation of the *Foxp3* locus or by potentiation of TGF- β signaling). Second, RA can inhibit the expression of the IL-6 receptor, thus

dampening the sensitivity to the inhibitory effect of this cytokine; this mechanism has been demonstrated by Xiao et al. and ourselves (Xiao et al., 2008; Hill et al., 2008). Third, RA can shut down the synthesis of cytokines (IFN- γ , IL-4, and IL-21), which in concert have an inhibitory effect on FoxP3 induction. This is the mechanism shown in our previous paper (Hill et al., 2008), by microarray analysis demonstrating a wholesale shutdown of cytokine genes by RA, consistent with other reports (Elias et al., 2008; Takaki et al., 2008; Maynard et al., 2009).

At issue, then, is whether RA can affect FoxP3 expression in isolated naive T cells, independent of other cytokines or other cellular influences. Experimental conditions in which naive T cells are stimulated in the presence of IL-6, of dendritic cells, or of other antigen-presenting cells do not address the point because they may read out mechanism #2 (e.g., experiments in Maynard et al. [2009] and Mucida et al. [2007]). Some reports have shown direct responses of carefully isolated naive T cells (Xiao et al., 2008; Elias et al., 2008;

and Figure S1 from the letter of Mucida et al., 2009). Yet, these inductions are carried out in conditions of T cell activation that can readily lead to effector cell differentiation, and thus to IFN- γ , IL-4, and IL-21 production (over 4 days' culture). Indeed, cytokine-mediated inhibition of FoxP3 expression may well be at play in some of these “naive responder” experiments. The progressive inhibition of FoxP3 induction with increasing doses of anti-CD28, and its reversal by RA (Figure S1 in Mucida et al., 2009), can plausibly be interpreted as cytokine production that increases with costimulatory signals. In the report of Xiao et al. (Xiao et al., 2008), RA treatment leads to increased Smad3 phosphorylation, and a known effect of IFN- γ signaling is the inhibition of Smad3 phosphorylation (Ulloa et al., 1999).

Thus, although the direct effects of mechanism #1 remain a possibility (and one suggested by the existence of an RAR-responsive element in the body of Foxp3 [Takaki et al., 2008]), the interplay between RA and inhibitory cytokines mean that any claim that FoxP3 induction